THE IMPACT OF HYDROXYAPATITE ON ALKALINE PHOSPHATASE ACTIVITY AND MINERAL DEPOSITION OF DENTAL PULP STEM CELLS USING A DOUBLE ANTIBIOTIC PASTE LOADED METHYLCELLULOSE CARRIER

by

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LIST OF ABBREVIATIONS

Alkaline phosphatase…….ALP

Calcium hydroxide…….$Ca(OH)_2$

Chlorhexidine ……......CHX

Dental pulp stem cells……...DPSCs

Double antibiotic paste……DAP

Ethylenediamine tetra-acetic acid …….……EDTA

Hydroxyapatite……...…HA

Methylcellulose……..MC

Mineralized trioxide aggregate……MTA

Regenerative endodontic procedures……..……REPs

Sodium hypochlorite…….……NaOCl

Triple antibiotic paste …….....TAP

INTRODUCTION

Immature permanent teeth are susceptible to pulpal and periapical infections through trauma, caries, and anatomic anomalies.[1] Dental trauma occurs frequently in children, with a 46% incidence of trauma to the primary and/or permanent dentition and a 22% incidence of injury to permanent dentition in children.[2] Population based studies have shown that the majority of these trauma cases occur to the incisors.[3] Dental trauma can often lead to pulpal necrosis, and if symptomatic, severe pain can occur with a significant impact on an individual's quality of life such as loss of the tooth.[4] With pulpal necrosis of an immature tooth, infection of the root canal occurs prior to completion of root formation. This produces root dentin that is thinner and shorter and an incompletely formed root apex. Structurally, this is problematic as the tooth is more susceptible to fracture.

In the past, the traditional way to treat necrotic immature teeth with incompletely formed roots was by utilizing the technique known as apexification.[5] In this technique, calcium hydroxide ($Ca(OH)_2$) is placed in the root canal system inducing the formation of a hard tissue barrier in the vicinity of the open root apex. Actual root formation is not achieved, but rather the presence of a calcified mass that permits filling of the root canal system against a hard tissue barrier of osteodentin. Although initially successful at four-year follow-up, this technique does not address the reduction in fracture strength that remains due to exposure of $Ca(OH)_2$, an alkaline substance, to acid proteins in the organic matrix surrounding the root canal system.[6] More recently mineral trioxide aggregate (MTA) and other bioceramic materials have been utilized in place of $Ca(OH)_2$ to create a hard tissue barrier in the apical area. Although MTA has improved properties over $Ca(OH)_2$, it is important to note that apexification with neither material permits

further root development in root length or width,[7] thereby not improving fracture resistance of an immature tooth with an incompletely formed root. While there is some evidence in the literature that newer bioceramics such as EndoSequence Root Repair Material have been used successfully in apexification cases with continued root growth, this evidence is limited to case reports and warrants further review.[8]

To address these shortcomings, recent advances in endodontics have produced techniques collectively referred to as regenerative endodontic procedures (REPs). The primary goal of these procedures is to have the tooth free of infection, asymptomatic, and permit bony healing of the apical periodontitis. Tertiary goals include regeneration of pulp-like tissue that can allow continued root formation to directly improve strength and fracture resistance. Ideally, the pulp-dentin complex is regenerated, and pulp revascularization permits normal responses to sensibility testing.[9] In cases of immature teeth with necrotic pulps, the goal is root development with apical closure.[10] Recently, tissue engineering has been a major focus of REPs, with the use of stem cell therapy, scaffold implant and placement of growth factors.[11]

REGENERATIVE ENDODONTIC PROCEDURES

Triple antibiotic paste (TAP) consisting of equal parts metronidazole, ciprofloxacin and minocycline has been utilized as an intracanal medicament for endodontic disinfection since the mid-1990s.[12] Chemical debridement through the use of localized antibiotic delivery is paramount to achieving success in REPs since these procedures typically involve little-to-no mechanical debridement.[13] A case study published in 2004 showed an immature necrotic tooth exhibited revascularization after treatment with TAP.[14] Since the publication of this case report, these results have been

replicated in several studies.[15-17] Research has shown, however, that the presence of minocycline contributed to tooth discoloration. To overcome this issue, a double antibiotic paste comprised of metronidazole and ciprofloxacin (DAP) was proposed[18, 19]and used successfully in regenerative endodontics.[20] Clinically used concentrations of TAP, DAP, sodium hypochlorite (NaOCl) and chlorhexidine (CHX) were found to have toxic effects on stem cells of the apical papillae.[21, 22] Research at this institution has been performed on finding the ideal concentration of DAP that exhibits a minimal effect on dental pulp stem cells (DPSCs) while sufficiently treating established biofilms. These studies found that DAP concentrations ranging from 0.125 to 1 mg/mL significantly reduced the bacterial biofilm while not having any significant effects on DPSC proliferation.[20, 23-25] Other studies, however, have noted that concentrations exceeding 0.125 mg/mL[20] and 0.3 mg/mL[26] negatively affect the proliferation rate of dental pulp cells. Additional research at Indiana University supports literature suggesting cytotoxic effects of clinically used concentrations of DAP and TAP on stem cells of the apical papillae and DPSCs.[25] Our overall research goal is to determine the appropriate concentration of DAP that will exhibit maximal antibacterial effects while minimizing any negative impact to stem cells.

Published reports have focused on evaluating the chemical structure and strength of dentin after regenerative endodontic procedures using DAP and TAP. One study reported a reduction in the phosphate/amide I ratio after dentin was treated with DAP or TAP.[27] In this study, evidence of degradation of collagen and demineralization of radicular dentin after exposure to DAP or TAP was shown.[27] These results suggest that DAP and TAP exhibit a demineralization effect and formation of a collagen rich matrix

on the radicular dentin. The authors hypothesize that the embedded collagen fibers and growth factors serve to enhance attachment and growth of host stem cells necessary for pulp regeneration.[27]

Traditionally, saline or sterile water have been used as a carrier for application of antibiotic pastes. Ongoing research at Indiana University has focused on utilizing methylcellulose (MC), a biocompatible, water-based hydrogel, for delivery of antibiotic paste in order to provide a clinically acceptable consistency.[28] The addition of MC allows for a more precise placement of antibiotic paste. Additional findings from the same study found that adding MC to antibiotic pastes minimizes some of the negative effects of these medications, such as a reduction in microhardness.[28] Further studies have sought to develop protocols for delivering concentrations of antibiotic pastes that are low enough as to not damage DPSCs, but also high enough to provide sufficient antibacterial effects.[29-31] Although these results have helped form the American Association of Endodontists' Clinical Considerations for a Regenerative Procedure,[32] in vivo results have been less consistent.

DENTAL PULP STEM CELLS

Since the early 2000s, much research has focused on the potential role of dental stem cells in tissue regeneration. Stem cells, in general, are undifferentiated embryonic or adult cells with the capacity to undergo continuous division.[33] Dental stem cells (DSCs) is a collective term used to refer to human dental stem/progenitor cells that have been isolated from various dental, periapical and periodontal sources.[33] With being able to isolate these cells, research has now focused on utilizing different types of DSCs, such as stem cells from the apical papilla (SCAP), for use in regenerative endodontic treatment.[34,

[35] Animal studies have shown that when dental pulp stem cells (DPSCs) are transplanted into immunocompromised mice, a dentin and pulp-like complex is formed.[36]

HYDROXYAPATITE

The extracellular matrix of bone is composed of an organic and inorganic matrix. The organic matrix, known as osteoid, is comprised of approximately 90% Type I collagen. This matrix is mineralized by hydroxyapatite (HA), which has the stoichiometric formula $Ca_{10}(PO_4)_6OH_2$.[37] The calcium to phosphorus molar ratio varies in bone mineral depending on the age, species and type of bone.[37] This ratio is important and has a direct impact on the rate of resorption within bone. Biomimetic precipitation methods have been utilized to create collagen and HA scaffolds that mimic the molecular structure of bone.[37] These scaffolds provide surface area on which DPSCs are able to bind.[38] This creates an osteoinductive activity that stimulates osteoblasts to form new bone.[39] The use of nano-HA in its nano-crystalline form has generated much interest in the field of dentistry. Some studies have reported that adding small amounts of nano-HA to mineral supplements for sports activities can lead to a decrease in tooth erosion.[38, 40] One beneficial aspect of nano-HA is its ability to cross cellular membranes. This has promising clinical implications allowing for targeted delivery of drugs within an encapsulated nano-HA scaffold.[41] Recent studies using collagen-HA scaffolds have shown increased attachment and proliferation of mesenchymal stem cells and human pulp dental stem cells[42] as well as improved osteogenic differentiation.[43] Additionally, some studies have focused on developing protocols for adding a collagen-HA scaffold to DAP in regenerative endodontic procedures. Several case reports have shown that HA provides calcium and phosphate ion sources for the formation of hard tissue. These authors note

that by adding HA to DAP, disinfection and hard tissue repair can both occur.[44]

PRELIMINARY DATA

Data from studies conducted by Sabrah et al. demonstrate that concentrations of DAP and TAP ranging from 0.125 to 10 mg/mL have similar antibacterial effects on established biofilms.[20] Depending on the assay performed, no cytotoxic impact on DPSCs was found with concentrations of DAP less than 0.5 mg/mL (for LDH assays) or 0.125 mg/mL (for WST-1 assays). Additionally, 0.125 mg/mL of either medication exhibited a significant antibacterial effect with no cytotoxic impact on DPSCs. Dentin samples pretreated with DAP had a longer residual antibacterial effect than TAP at equal concentrations.[20]

Additional studies at this institution have shown that adding a MC carrier to antibiotic pastes can improve the microhardness compared to antibiotic treatment alone.[28] Recent studies have added MC carrier to varying concentrations of DAP to analyze DPSC proliferation and differentiation. These data note that 1 mg/mL DAP in MC had similar efficacy on DPSCs when compared to the clinically widespread $Ca(OH)_2$.[30] When DAP concentrations were increased, proliferation and mineral deposition assays exhibited a decrease inferring that DAP is toxic to DPSCs in higher concentrations. However, 1 mg/mL DAP showed an increase in proliferation and differentiation compared to control. Consequently, for this study we will use 1 mg/mL DAP in a MC carrier with varying concentrations of HA.

A unique feature of nano-HA is its ability to bind to bone without inducing a toxic or inflammatory response.[39] This is one reason why research using HA has profound clinical implications for regenerative medicine. In one study analyzing the addition of

nano-HA to sports drinks, 0.25% nano-HA helped prevent dental erosion after acid exposure.[40] In an unpublished study at this institution, assay tests measuring differentiation and mineral deposition showed the greatest activity in the groups with 0.5% HA or 1.0% HA.[45] Everhart et al. demonstrated increased proliferation of DPCS after 3-day treatment with the medicaments (1.0% DAP with MC) and varying concentrations of nano-HA.[45] Specifically, the researchers used 0.25%, 0.5% and 1.0% nano-HA in 1 mg/mL DAP + MC and then performed an MTS assay (Promega CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay), which is based on cell metabolic activity, to quantify cell proliferation. A robust response was noted across all experimental groups with the authors concluding that the experimental groups did not negatively influence proliferation of DPSCs. In addition to proliferation, the researchers examined the impact of nano-HA on DAP with MC by measuring ALP activity and mineral deposition by DPSCs after culture in osteogenic media for 7 days. ALP is a commonly used osteogenic marker that evaluates differentiation of stem cells into osteogenic lineage through the conversion of p-nitrophenol phosphate to p-nitrophenol.[46] ALP was increased in 7-day cultures, suggesting no cytotoxicity effects of the medicaments over 7 days. While the increase in ALP was significant in this timeframe, the mineral deposition assay produced only modest differences between the experimental groups. Mineral deposition was measured using the Alizarin Red S assay. This assay is used to quantify calcium formation by mature osteoblasts. Alizarin Red S, an anthraquinone dye, binds to calcium ions in the cell and stains these cells red. The stain is then extracted using 1% cetylpyridinium chloride (CPC) and samples are analyzed using spectrophotometry.[47]

Based on these preliminary results, we hypothesized that altering the treatment exposure time of DPSCs to medicament with nano-HA could result in an increase in mineral deposition. We planned to test the effects of 3-day treatment time, after which ALP and mineral deposition will be measured 9 days post-treatment. Increasing or decreasing the exposure time could allow for cells that have undergone proliferation in the presence of HA to undergo osteogenic differentiation, resulting in mineral deposition, while minimizing any possible cytotoxic effects of the medicaments. This could produce more bone formation and improve clinical outcomes. These studies will enable us to determine if exposure time to medicaments is important to the physiological outcomes.

OBJECTIVE

The objective of this study is to evaluate the effects of HA in a DAP loaded MC carrier on the differentiation and mineral deposition of DPSC over time.

NULL HYPOTHESIS

The addition of HA nanoparticles to a DAP loaded MC carrier will have no effects on the differentiation and mineral deposition of DPCS over time.

ALTERNATIVE HYPOTHESIS

The addition of HA nanoparticles to a DAP loaded MC carrier will increase the differentiation and mineral deposition of DPSC over time.

REVIEW OF LITERATURE

HISTORY OF ENDODONTICS

The ancient origins of dentistry hark back to approximately 7000 B.C. in the Indus Valley Civilization where excavations of *Homo sapiens* noted evidence of treatment for tooth related problems.[48] However, it wasn't until 5000 B.C. when a Sumerian text described "tooth worms" as the cause of dental decay.[49] The earliest known reference to a dental practitioner can be traced to Hesy-Re, an Egyptian scribe, whose tomb circa 2600 B.C. reads "the greatest of those who deal with teeth, and of physicians".[49]

In the Middle Ages, the first book devoted entirely to dentistry entitled *Artzney Buchiein wider allerlei kranckeyten und gebrechen der tzeen (Medical Booklet for All Sorts of Diseases and Injuries to the Teeth)* was published in German in 1530.[50] In 1687, Charles Allen published the third edition of the first book in English devoted entirely to dentistry.[51] In 1700, Anton von Leeuwenoek, the "father of modern microscopy" incorrectly attributed tooth pain to the gnawing of worms from the egg of a small fly.[52] Although this assumption is now known to be incorrect, it is perhaps the first explanation of dental pain being caused by an external source.

Twenty-eight years later, Pierre Fauchard, the "founder of modern dentistry" wrote *The Surgeon Dentist* in 1728.[51] In this book, Fauchard described pulp extirpation as well as the opening of teeth to relieve abscesses and permit drainage of pus. After the tooth was left open for two to three months, Fauchard described filling the pulp chamber with lead foil.[51] Fauchard's thinking helped give rise to the "empirical era" as belief in "tooth worms" became less supported. In 1725, Lazare Riviere described the use of oil and cloves for sedative purposes.[53] In 1756, a German dentist named Phillip Pfaff

described pulp-capping using a concave piece of gold of lead over the exposed pulp area. This technique was likely an improvement over Fauchard's direct capping method.[54]

The earliest record of endodontic therapy in the United States was performed by Robert Woofendale, a dentist who came to New York in 1766. Woofendale's technique included cauterizing the pulp with a hot instrument and stuffing the pulp chamber with cotton.[55] No recorded attempt was made to fill the canals.[56] At that time, no emphasis was placed on instrumenting or removing the radicular tissue. Rather, this tissue was often covered with oils from cinnamon, cloves, or turpentine to provide relief.[51]

The 1800s brought about what is now referred to as the Vitalistic Era. In 1805, the issue of vitality with pulp treatment was first recognized by J.B. Gariot when he noted that destroying the pulp in the chamber does not destroy the vitality of the tooth.[57] Edward Hudson, an Irish dentist practicing in Philadelphia, is credited with being the first to place fillings in root canals.[51] Hudson designed his own pluggers and used gold foil to pack the canals.[56] Note, however, that emphasis was not placed on treating the canal. In 1819, John Callow of London credited Charles Bew with describing the path of blood flow through the apical foramen and into the pulp. Bew is credited with adding more credence to the "vitalistic" theory that many believed at this time.[57]

Leonard Koecker, a successful dentist during his time, wrote "Principles of Dental Surgery" in 1826 which became a standard for 50 years.[51] Koecker believed that when the pulp was destroyed by disease or artificial means, the pulp in the chamber died. This in turn produced a foreign body that required extraction to prevent inflammation and death of surrounding tissues.[51] To avoid losing the tooth, Koecker first described pulp capping by cauterizing exposed pulp and protecting it with lead foil in 1820.[58] This

technique was very similar to what Plaff described in 1756, however Koecker also believed that healthy, living tissue could not remain viable next to dead tissue.

In 1829, further credence was given to the "vitalistic" theory when S. S. Fitch outlined its doctrines in *System of Dental Surgery*. Fitch believed that the crown of the tooth was nourished by the dental pulp, whereas the roots of the teeth were supported by pulp membrane on the interior and the alveolar membrane on the exterior.[51] This theory stated that when the pulp was removed, only the crown lost vitality whereas the roots remained vital via the periodontal membrane. As such, the crowns of teeth were removed after pulp extirpation leaving the roots in place.

In 1836, Shearjashub Spooner recommended arsenic trioxide (a protoplasmic poison) for pulp devitalization prior to removal.[58] In 1839, Baker was credited in the *American Journal of Dental Science* with the first published account of pulpal extirpation, canal shaping and cleaning, and root canal filling.[59] In 1850, W.W. Codman claimed in the *Boston Medical and Surgical Journal* that pulp capping aimed to produce secondary dentin at the site of the pulp exposure.[51] In 1851, S. P. Hullihen described an operation to deplete a congested pulp by drilling through the gum and labial alveolar plate into the pulp chamber to induce hemorrhage. He termed this operation rhizodontrypy.[56]

The mid-to-late 1800s saw the advent of many dental supplies still in use today for endodontic treatment. Until the 1870s, dental depots did not carry instruments for root canal treatment. In 1838, Edwin Maynard filed a watch spring to the fineness of a horsehair for use as a root canal broach. This permitted more accessible treatment of posterior teeth as well as teeth with small canals.[53] Gutta-percha was introduced in 1847

by Edwin Truman as a filling material for root canals and denture bases.[51, 58] In 1864, S.C. Barnum devised a thin, rubber sheet to isolate the tooth during endodontic treatment.[51] G.A. Bowman is credited with co-inventing the rubber dam clamp forceps in 1873.[56] In 1867, Magitot suggested using an electric current to test pulp vitality.[60] In 1886, George Evans described an instrument used to disinfect and dry the canal prior to filling.[56] In 1891, J.S. Marshall proposed using the electric pulp tester to determine the vitality of teeth.

The *Dental Cosmos* published an article in 1878 by G.O. Rogers suggesting that pathogenic organisms might be the most common causes of disease of the dental pulp. Rogers concluded that successful treatment required disinfection of the pathogenic organisms.[61] This belief in the importance of recognizing the pathogenicity of bacteria led to the birth of septic theory.[57] In 1879, during this transition from vitalism theory to septic theory, Charles S. Tomes attempted to combine recent understanding of pathogenicity with the theory of vitalism. Tomes argued that dentin lost vitality under septic or arsenical influence and contaminated cementum, which also became infected and disrupted the periodontal membrane.[57]

In 1882, Arthur Underwood added additional support for the septic theory. He surmised that pathogens are responsible for the suppuration of the pulp and development of alveolar abscess. Underwood then went on to propose that if the canal space was sterilized with use of antiseptic agents, then disease could be prevented[61] regardless of whether the pulp was vital. This thinking led to the use of caustic germicides by clinicians for over 30 years.[57] Examples of germicides include arsenic, formalin, chlorophenol, sodium dioxide and sulphuric acid, to name a few.

In 1883, G.A. Mills described in *Dental* Cosmos a pulp extirpation procedure that involved a tapered wooden point dipped in creosote or carbolic acid being driven to the apex with the quick blow of a mallet.[61] In 1885, Lepkoski replaced arsenic with formalin to "dry" the non-vital pulp stumps that remained after extirpation[62] without the caustic side effects of arsenic. Camphorated chlorophenol was introduced as a sterilization medication by Otto Walkhoff in 1891.[61] Eight years later, Hermann Prinz introduced this medicament to American dentists in 1899.[58] An article published in the *Dental Cosmos* in 1886 by Dr. Evans outlined an instrument for disinfecting devitalized teeth with heat. This instrument became known as the Evans root drier.[63]

Before the turn of the century, prosthetic restorations, such as the Davis or Richmond crown, became increasingly popular. This created greater demand for endodontic therapy as these procedures often required the use of a dowel in the root canal.[61] Frequently these techniques were performed with little regard for aseptic technique. In 1888, W.D. Miller, an American dentist residing in Berlin, proposed the bacteriological basis for root canal treatment by correctly describing the formation of abscesses as a continuation of pulpal infection.[60] Miller advocated for sterilizing dentin for thirty minutes using antiseptic solutions.[61] Furthermore, he stressed that any organ contacted by bacteria could produce a metastatic abscess if any points of reduced resistance were present.[64]

In 1895 in the Bavarian city of Wurzburg, Konrad Wilhelm von Roentgen made the lasting impact on medicine by discovering the X-ray.[58] Only a few weeks later, Otto Walkhoff took the first dental radiograph,[58] however radiographs were not commonly used in dentistry until after 1910.[56] Shortly after Roentgen's discovery and Walkhoff's

dental radiograph, Dr. C Edmund Kells began to use X-rays in his dental practice in New Orleans.[61] During his time, Kells helped popularize the technology which permitted dentists to evaluate their root canal post-operatively. Sadly, he died in 1928 of cancer caused by early experimentation with X-rays.[61] In 1908, Dr. Meyer L. Rhein developed a technique utilizing wire and X-rays to determine canal length and obturation quality.[58] Around this same time, G.V. Black proposed a measurement control to avoid overfilling teeth.[58]

Around 1904, formocresol, a mixture of tricresol and formalin, was introduced by John Buckley and is still in use today.[59] In 1906, H.S. Vaughan of New York utilized infiltration anesthesia to anesthetize the pulp prior to treatment.[56] The development of this technique was monumental because it started the beginning of the painless era in dentistry.

In 1909, E.C. Rosenow, a mentee of Frank Billings, developed the focal infection theory by demonstrating streptococci present in many diseased organs and could spread via the bloodstream.[62] Shortly after, Mayrhofer linked pulpal infection with microorganisms having found streptococci in approximately 96% of the cases reviewed.[65] The following year, events transpired that nearly brought the practice of endodontics to a halt in western culture. In 1910, an English physician and pathologist named William Hunter gave a lecture titled *The Role of Sepsis and Antisepsis in Medicine* which was subsequently published in *Lancet*.[66] In his lecture, Hunter was extremely critical of dentistry, and specifically the practice of prosthetic dentistry in largely septic conditions. He claimed that gold crowns were “a mausoleum of gold over a mass of sepsis” and was critical of poorly formed prosthetic restorations. Even so, his lecture was widely

interpreted as an indictment against the pulpless tooth. Consequently, for nearly 40 years dentists continued to extract any devitalized teeth and those who did were known as the "one hundred percenters".[58]

The silver lining during this period of dentistry is that it caused several clinicians to improve their aseptic techniques.[56] In 1912, Rhein provided one of the first rebuttals to the focal infection theory and tried to get clinicians to favor aseptic techniques such as rubber dam utilization.[66] Around this time, dental research became more focused on biologic principles and the use of medicaments in treatment. In 1920, B.W. Hermann of Germany began using a $Ca(OH)_2$ mixture for filling foot canals, and later for various endodontic procedures. Herman supported his research with histological data and believed $Ca(OH)_2$, called Calxyl, was superior to other substances at the time that were cytotoxic and would cause the formation of various lesions.[53] In 1929, Balint Orban commented on the healing power of the dental pulp after noting under histologic evaluation that dental pulp harbors defense and repair cells.[66] Around 1930, treatment started to become less in favor of the techniques practiced by the "one hundred percenters." Increasingly, more conservative treatment was becoming widespread with improved radiographic systems, bacteriological culturing and greater emphasis on diagnosis and aseptic technique.[66] In 1931, Rickert and Dixon proposed their "hollow tube effect" hypothesis that if a void is left in a root canal filling, the space will fill with tissue fluid which, when broken down, can cause an inflammatory reaction.[60]

The late 1930s brought about the beginning of the scientific era which helped marginalize the focal infection theory in dentistry. In 1937, Logan proposed that bacteria can be present in normal tissue without having any pathological significance.[55] In 1944,

Fred Adams and Louis Grossman were instrumental in advocating for the use of penicillin in root canal treatment. Grossman, known as the "father of modern endodontics," suggested using a nonaqueous solution of penicillin and later sterilized root canals with paper points impregnated with the antibiotic.[66]

Not until the late 1940s or early 1950s did the dental and medical professions recover from the focal infection theory and endodontic treatment of molars was taught once again in dental schools.[67] In February 1943, 20 individuals met in Chicago and formed the American Association of Endodontists.[66] Harry B. Johnston coined the term "endodontia" by combining the Greek words "endon" (within) and "ho dontas" (a tooth). The first journal devoted entirely to endodontics, *The Journal of Endodontia,* was published in 1946.[66] In 1956, the American Board of Endodontics was formed after conferring with the American Dental Association's Council on Dental Education.[66] In 1963, over 200 American dentists were limiting their practice to endodontics. This same year the American Dental Association formally recognized endodontics as a dental specialty, and in 1965 the first Diplomates received certification.[66]

THEORY OF ENDODONTICS

A major contribution to the field of endodontics came in 1965 when Kakehashi, Stanley and Fitzgerald published their landmark study on germ-free rats. These researchers illustrated the role of bacteria in the progression of pulpal inflammation to apical periodontitis.[68] In their study, germ-free rats did not develop apical periodontitis following pulp exposure, whereas conventional rats with normal oral flora developed pulpal necrosis and apical periodontitis. They concluded that the presence of a microbial flora is a major determinant in the healing potential of dental pulp.[68]

Adding to the belief that bacteria play a role in dental pathology, in 1974, Bergenholtz noted that the majority of traumatized teeth with intact crowns and necrotic pulps had primarily had polymicrobial microorganisms present.[69] In 1976, Sundqvist elegantly wrote in his thesis that necrotic pulps contain a variety of microorganisms with a propensity for anaerobic bacteria.[70] In 1981, Moller expanded on the work of Kakehashi, Stanley and Fitzgerald and noted that bacteria are an etiologic factor for apical periodontitis. Moller found that devitalized noninfected teeth in monkeys produced no periapical pathology, whereas necrotized and infected pulps produced periapical lesions.[71] This microbiological study was monumental because it added further support for the understanding that necrotic tissue would not necessarily produce apical lesions, but rather microorganisms are required.

Endodontic research from this era led to the understanding that endodontic and periapical pathology can be attributed to the presence of microorganisms. Microorganisms, already present in the oral flora, can gain access to the root canal system by way of caries and trauma. The aim of treatment, therefore, is to reduce the microbial insult to a level that will permit adequate healing. In 1955, G.G. Stewart outlined endodontic therapy into three phases: chemomechanical preparation, microbial control and obturation of the root canal system.[72] Stewart emphasized that thorough chemomechanical cleansing of the root canal system is arguably the most important aspect of treatment.

In 1967, Grossman expanded on Stewart's three phases of therapy and presented thirteen principles of endodontic treatment:

1. Aseptic technique

2. Retain instruments within the root canal system
3. Never force instruments apically
4. Enlarge the canal space to allow obturation material
5. Continuous irrigation throughout treatment
6. Irrigation solution should remain within the root canal system
7. Fistulas do not require special treatment
8. Confirm negative culture prior to obturation
9. Obtain hermetic seal of the canal system
10. Obturation material should not be irritating to the periapical tissues
11. Provide adequate drainage for acute alveolar abscess
12. Avoid infections into infectious areas
13. Apical surgery may be required to promote adequate healing

That same year, Schilder promoted the importance of a dense, three-dimensional obturation. He argued that the objective of obturation should be the total, three-dimensional filling of the root canal system, including all accessory canals.[73] The technique described by Schilder involved placing a master cone followed by adding incremental segments of warm gutta percha with vertical condensation.[73] In 1974, Schilder wrote that in order to achieve predictable success in endodontic therapy "root canal systems must be cleaned and shaped – cleaned of their organic remnants and shaped to receive a three-dimensional hermetic filling of the entire root canal space."[74]

In 1983, based on histologic examination, Pitt Ford wrote that it is necessary to fill the root canal, but not necessary to achieve a hermetic seal.[75] Rather, he argued that value of an obturation material centers on three concepts. First, the filling ensures a

reduction in space available for bacterial colonization. Second, the filling prevents microbial contamination of the apex from saliva after extirpation of the pulpal tissue. Third, a filling prevents movement of microbes along the root canal system.[75]

MECHANICAL INSTRUMENTATION

Mechanical instrumentation is the first phase of endodontic treatment and serves to remove debris, permit penetration of irrigation to the apex and prepare the canal for obturation.[76, 77] Mechanical and biologic objectives need to be met in order to ensure successful endodontic therapy. Mechanical objectives include preparation of all surfaces with the original anatomy centered in the final preparation. In fact, the most difficult area to maintain the original anatomy and clean is the apical area.[78] It is imperative to sufficiently prepare the canal for irrigation and obturation, without procedural errors that can weaken the tooth and damage surrounding structures.[79]

Biologic objectives of mechanical instrumentation include removal of sufficient pulp and dentin to create adequate space for the delivery of irrigants. Salzgeber and Brilliant noted that a minimum apical file size of 30 was needed to permit sufficient flow of irrigant to the apex.[77] Schilder suggested preparations with a uniform and continuous taper, however his suggestion was more geared towards facilitating obturation.[74] Furthermore, mechanical instrumentation helps reduce the microbial concentration by 100-1000 fold.[80] Although this reduction in bacterial concentration is helpful, it is important to note that instrumentation leaves 35% of the canal walls untouched.[81] Consequently, another mode of treatment is required to remove the debris and eradicate any microorganisms.

CHEMICAL IRRIGATION

The role of irrigation in endodontic therapy is to flush out debris and reduce the microbial load. The root canal system is full of anatomical irregularities including isthmuses and lateral canals[82] that are difficult to treat with mechanical instrumentation alone. While no product satisfies all criteria, the ideal irrigant has broad antimicrobial properties, is highly effective against anerobic and facultative organisms, inactivates lipopolysaccharides, dissolves vital and necrotic tissue, and prevents or dissolves the formation of a smear layer.[83] The most frequently used irrigant is NaOCl, commonly considered the gold standard in endodontics. NaOCl is successful due to its antibacterial capacity against planktonic and established biofilms and its ability to dissolve necrotic and vital tissue.[82, 84]

The mode of action of NaOCl is multifaceted. In addition to its saponification, neutralization and solvent actions, NaOCl forms hypochlorous acid and hypochlorite ions that lead to amino acid degradation and hydrolysis.[85] Furthermore, its high pH (pH > 11) disrupts cytoplasmic membrane activity due to irreversible enzymatic inhibition, alterations in cellular metabolism and phospholipid degradation.[85] Although NaOCl is close to the ideal irrigant, it lacks substantivity and does not dissolve the mineralized component of the smear layer. Consequently, many clinicians turn to adjunct irrigants to satisfy these goals.

Chlorhexidine gluconate (CHX) is a biguanide that is easily soluble in water and very stable. It is a wide-spectrum antimicrobial rinse that is bacteriostatic and bactericidal against gram-positive and gram-negative bacteria.[86] Studies have shown that a 10 minute rinse with CHX is the most effective means to eliminate *E. faecalis* from the canal system.[87] Furthermore, CHX has been shown to exhibit substantivity by binding to dentin

up to 48 days after exposure.[88] Drawbacks include the inability to dissolve organic substances[89] and the possible formation of a carcinogenic precipitate, parachloroaniline, when reacting when NaOCl.

Ethylenediamine tetra-acetic acid (EDTA) is an aminopolycarboxylic acid and a chelator that is used to remove the mineralized portion of the smear layer during treatment. Studies have found that a 1-minute rinse with EDTA is sufficient, however prolonged use can lead to excessive dentin erosion.[90] Additionally, care must be taken when using EDTA in combination with NaOCl as EDTA can reduce the efficacy of NaOCl, due to a decrease in the active chlorine content, and possibly affect its antimicrobial efficacy.[91]

OBTURATION

Upon completion of the cleaning and shaping, the root canal system is ready for obturation with a three-dimensionally adapted filling.[73] Traditionally, gutta-percha (GP) has been used to obturate canals. Gutta-percha is the trans-isomer of polyisoprene (rubber) and exists in two crystalline forms, alpha and beta. Gutta-percha cones are composed of zinc oxide (65%), gutta-percha (20%), and other materials including waxes, resins, and metals.[92] Various filling techniques such as lateral condensation and vertical condensation using gutta-percha exist, and research has shown no differences in outcomes between the two techniques.[93]

Prior to placing the obturation material, a root canal sealer is added to the canal walls to seal the space between the dentin and obturation material. There are several ideal properties of a root canal sealer, some of which include establishing a hermetic seal, being radiopaque, permit no shrinkage of setting, is bacteriostatic, exhibits slow setting

time, is insoluble in tissue fluids, is biocompatible, is soluble in a common solvent and exhibits tackiness when mixed.[94] No sealer presently available fulfills all these criteria. Some of the most popular sealers currently used are zinc oxide-eugenol based sealers, resin-based sealers, and calcium silicate-based sealers. While there are differences among all available sealers, all sealers exhibit cytotoxicity when freshly mixed and care should be taken to avoid extrusion into the periradicular tissues.[95] Overall, each type of sealer has its merits and limitations, so ultimately it is up to the clinician to decide when one type of sealer is indicated over another.

MICROORGANISMS

Microbial infection of the root canal system can lead to an inflammatory condition of the periapical tissues called apical periodontitis. The role of bacteria in apical periodontitis was confirmed in the classic study by Sundqvist who found bacteria only in the root canals of teeth radiographically exhibiting apical periodontitis.[70] Generally, the primary goal of root canal treatment is to prevent or otherwise heal apical periodontitis.[96] While much of the body is colonized by bacteria, the dental pulp and surrounding tissues are typically sterile. Bacteria are able to gain access to the untouched or previously treated root canal system directly via dental caries or fracture, or indirectly via microcracks.[69] Regardless of the route of infection, endodontic infections are frequently polymicrobial and reside in organized communities known as biofilms.[97] Biofilms are sessile, multicellular microbial communities characterized by the formation of an extracellular matrix of polymeric substances.[98, 99] The ability to form biofilms is considered a virulence factor. These communities are characterized by their diverse metabolic activity, concentration gradient, exchange of genetic material, and quorum

sensing.[100] Consequently, these communities are able to effectively act as a group thereby making their eradication more troublesome than sessile bacteria.

Various bacterial species colonize endodontic infections. Endodontic bacteria frequently fall into nine phyla: Firmicutes, Bacteroidetes, Spirochaetes, Fusobacteria, Actinobacteria, Proteobacteria, Synergistetes, TM7 and SR1.[101, 102] Commonly isolated bacteria include both facultative and obligate anaerobes such as members of the *Streptococcus, Enterococcus, Prevotella,* and *Porphyromonas* species. Primary infections are infections of the necrotic pulp tissue in an untreated tooth. These infections are characterized by equal numbers of gram-positive and gram-negative species with more obligate anaerobes than aerobes present.[103] Persistent infections are infections that have resisted antimicrobial procedures and endodontic therapy. Secondary infections are infections that have contaminated the obturated root canal system at some point post-treatment. While the microorganisms colonizing primary infections are more diverse, secondary infections are typically colonized with far fewer species.[102] Oftentimes *Enterococcus faecalis,* a gram-positive facultative anaerobe, is found to be associated with secondary infections.[104] This is partly due to virulence factors such as the presence of a proton pump[105] and ability to survive for long periods in dentinal tubules.[106]

IMMATURE TEETH WITH PULPAL NECROSIS

Treatment of the immature tooth with pulpal necrosis presents several challenges for the clinician. Radiographically these teeth present with thin dentinal walls that are at risk of fracture[107] and an apex that is often blunderbuss in shape.[19] Cleaning and shaping of these cases can be difficult, and obturation poses the risk of extrusion of material into the periapical tissues.[108] Treatment options for immature teeth with pulpal necrosis

include apexification and regenerative endodontic procedures. In cases with an immature, vital pulp that is irreversibly inflamed, apexogenesis procedures are preferred.

APEXOGENESIS

Apexogenesis is a vital pulp therapy procedure aimed to promote physiologic root end formation.[109] Techniques include pulp capping (either direct or indirect) and pulpotomy. Pulp capping aims to treat the exposed pulp with a restorative material that will permit formation of reparative dentin and continued maintenance of the vital tissue. Prognosis is dependent on how quickly restorations are placed after exposure,[110] as well as site of the exposure, patient age, and material used.[111] Traditionally, $Ca(OH)_2$ has been the restorative material of choice, however nowadays more biocompatible materials such as MTA and Biodentine are preferred.

Pulpotomy procedures involve partial or full removal of the coronal pulp in order to preserve the vitality of the radicular pulp. The extent of coronal pulp tissue removed depends on the level of inflammation present and the treatment goal. A partial pulpotomy, commonly referred to as a Cvek pulpotomy, involves removal of the coronal pulp tissue to the level of healthy pulp in an immature permanent tooth.[112] Originally $Ca(OH)_2$ was used to cover pulp tissue, however MTA and newer bioceramics have been used with predictable results.[113]

APEXIFICATION

Apexification is a procedure aimed to develop a hard tissue barrier in immature teeth with pulpal necrosis.[109] The first example of apexification in the literature involved placement of $Ca(OH)_2$ to stimulate the Hertwig epithelial root sheath to form an apical stop.[114] Repeated applications of $Ca(OH)_2$ are often required every few months, with the

barrier formation taking as long as 24 months to fully form.[115] Although $Ca(OH)_2$ has been used for decades for apexification procedures with predictable results, long term use has been shown to increase the incidence of root fracture.[6, 107] Current apexification techniques favor bioceramic materials such as MTA, Biodentine and EndoSequence Root Repair Materials. Studies have shown that these procedures can be completed in one visit with superior outcomes to $Ca(OH)_2$.[116]

REGENERATIVE ENDODONTICS

Per the AAE, regenerative endodontics are biologically-based procedures designed to physiologically replace damaged tooth structures, including dentin and root structures, as well as cells of the pulp-dentin complex.[109] The primary goal of regenerative endodontic procedures is resolution of symptoms and apical pathosis. Second and tertiary goals include increased root length and wall thickness and a positive response to pulp sensibility testing, respectively.[9] Significant research has poured into this field in recent years and it is increasingly becoming seen as an acceptable alternative to apexification for the treatment of necrotic immature permanent teeth.

Nygaard-Ostby provided the impetus for regenerative endodontics in the 1960s with his research on blood clots and the potential for revascularization in the root canal system.[117] He hypothesized that a blood clot could help stimulate healing in the root canal system, similar to how a blood clot stimulates healing in the alveolar bone following extraction. In 1966, polyantibiotic pastes were used in immature necrotic teeth to disinfect the canal system and promote root development.[118] Although intracanal bleeding was not intentionally induced in this study, resolution of signs and symptoms and continued root formation was noted. In 1971, Nygaard-Ostby performed a study

utilizing antibiotics for disinfection along with intracanal bleeding to show resolution of symptoms and continued root development.[119] Overall interest in the field dissipated until 2001 when Iwaya et al. presented a case report showing revascularization of an immature permanent tooth.[18] Additional excitement was generated in 2004 when Banchs and Trope published their case report showing pulp revascularization of an immature mandibular premolar that had radiographic and clinical signs of apical periodontitis along with a sinus tract.[14] Since the early 2000s, there has been an explosion of case reports showing successful regenerative endodontic treatment as well as translational research studying the role of tissue engineering in clinical treatment.

Technique for regenerative endodontic procedures focuses on minimal instrumentation with copious irrigant use. The aim is to prevent over-instrumentation of thin dentinal walls and avoid iatrogenic errors.[120] Choice of irrigant must balance the ability to properly disinfect the infected root canal system without impacting stem cell survival. Research has shown CHX to be toxic to stem cell survival[21] and its use is contraindicated in regenerative endodontic procedures. Rather, low concentrations of NaOCl have been found to provide sufficient disinfection while minimizing stem cell toxicity.[22] Additionally, EDTA has been recommended due to its ability to release growth factors in dentin.[121]

Case reports have helped outline protocols for successful completion of regenerative endodontic treatment. Iwaya recommended a double antibiotic paste (DAP) comprised of ciprofloxacin and metronidazole for disinfection of the root canal system.[18] In 2004, Banchs and Trope recommended adding minocycline to cirprofloxacin and metronidazole, forming a mixture referred to as triple antibiotic paste (TAP).[14] This was

based on the research of Hoshino et al., who found that this mixture eradicated infected dentin in vitro.[12] Although this concoction provides sufficient disinfection clinically, it also contributes to staining of teeth due to the presence of minocycline.[122] Regardless of the medicament used, sufficient contact time is warranted with studies showing that placement of an intracanal medicament for two to four weeks is needed.[123]

INTRACANAL MEDICAMENTS

To initiate disinfection of the root canal system in regenerative endodontic procedures, minimum instrumentation with copious irrigation is recommended.[120] After the first visit, an intracanal medicament is placed to provide enhanced antimicrobial activity. Various medicaments have been developed, each with their own benefits and drawbacks.

CALCIUM HYDROXIDE

Calcium hydroxide ($Ca(OH)_2$) was first developed circa 1920 by B.W. Hermann and has been frequently used in endodontic treatment since due to its broad antimicrobial properties.[124] $Ca(OH)_2$ has a high pH (approximately 11-12) and is able to diffuse into dentin tubules causing an increase in root dentin pH for up to three weeks.[125] $Ca(OH)_2$ works on direct contact, inhibits microorganism DNA replication, and reduces the cytotoxic response to lipopolysaccharide by destroying the lipid A moiety.[126] While it is effective against most bacteria in the root canal system, *E. faecalis* has shown resistance to $Ca(OH)_2$.[105] Regarding regenerative endodontic procedures, $Ca(OH)_2$ has shown increased stem cell survival and proliferation.[127] Additionally, it has shown reduced cytotoxicity against stem cells of the apical papilla when compared to antibiotic pastes in clinically used concentrations.[128]

TRIPLE ANTIBIOTIC PASTE (TAP)

Triple antibiotic paste was first formulated by Hoshino et al. who showed its efficacy in eradicating microbes from root canal dentin.[12, 129] Comprised of a 1:1:1 mixture of ciprofloxacin/metronidazole/minocycline, TAP's efficacy has successfully been demonstrated in preclinical models[130] and case reports.[14] Clinical issues have arisen, however, with studies noting that minocycline contributes to dental staining.[122] Additionally, studies have shown that it is more difficult to remove TAP from the root canal system at the second visit than adjunct intracanal medicaments.[131] This leads to concerns of residual TAP being cytotoxic to potential stem cells. Due to these drawbacks, alternative intracanal medicaments have been developed.

DOUBLE ANTIBIOTIC PASTE (DAP)

DAP, comprised of a 1:1 ratio of ciprofloxacin and metronidazole, was first recommended by Iwaya et al. after showing antimicrobial activity against endodontic pathogens.[18, 44] Research at this institution has supported the efficacy of DAP[23, 24] and its reduced cytotoxicity to stem cells.[20] These benefits, along with the omission of minocycline which can cause dental staining, have led to increased interest in DAP in regenerative endodontic procedures.

COMPONENTS OF REPs

In order to achieve the primary, secondary and tertiary goals of regenerative endodontics, a solid appreciation of tissue engineering is warranted. Successful tissue engineering requires three components: stem cells, growth factors, and a scaffold.[132]

STEM CELS

Stem cells are undifferentiated cells with the capacity for self-renewal and

differentiation. These cells can be classified as totipotent, pluripotent or multipotent depending on their differentiation ability. Stem cells relative to dentistry are also classified according to their location in the oral region. These include stem cells of the apical papilla (SCAP), dental follicle stem cells (DFSCs), dental pulp stem cells (DPSCs), inflammatory periapical progenitor cells (iPAPCs), bone marrow stem cells (BMSCs), periodontal ligament stem cells (PDLSCs), tooth germ progenitor cells (TGPCs), salivary gland stem cells (SGSCs), stem cells of human exfoliated deciduous teeth (SHED), oral epithelial stem cells (OESCs), gingival-derived mesenchymal stem cells (GMSCs) and periosteal derived stem cells (PSCs).[133] Stem cells involved in regenerative endodontics are primarily found in the apical hard and soft tissues, as well as the dental pulp and dental follicle. These include SCAP, PDLSCs, BMSCs, iPAPCs, DPSCs and DFSCs.[10] Studies have shown that mesenchymal stem cells that infiltrate the root canal space originate in the apical papilla and provide significant proliferation and differentiation capability.[34, 134] DPSCs are found throughout the dental pulp and localize in the cell-rich zone of Hohl next to the odontoblastic layer.[135] After localized injury, these cells form a distinct form of reparative dentin called "osteodentin."[136]

SCAFFOLD

The scaffold is an integral component of tissue engineering due to its ability to localize cells, release growth factors and undergo biodegradation over time.[137] Scaffolds can be comprised of natural or synthetic materials. Natural scaffolds include collagen, glycosaminoglycans, hyaluronic acid and dentin matrix.[138, 139] Synthetic scaffolds include HA/tricalcium phosphate, bioceramics, and hydrogels.[42, 140] Typically, the scaffold and growth factors in regenerative endodontic procedures are derived from the dentin, fibrin

clot and remaining pulp tissue, apical papilla and Hertwig's epithelial root sheath.[141] Recent interest has focused on autologous scaffolds, including platelet-rich plasma (PRP) and platelet-rich fibrin (PRF). These scaffolds are biocompatible, rich in growth factors, degrade over time, and form a three-dimensional fibrin matrix.[142, 143] Multiple case reports have been published supporting the success of PRF and PRP in regenerative endodontics.[142, 144-146]

GROWTH FACTORS

Various growth factors have been found embedded in the mineralized dentin matrix. These endogenous molecules play a role in cell signaling and are responsible for a variety of processes aimed at healing after dental injury.[147] Studies have found that dentin-derived growth factors play a role in the recruitment, proliferation and differentiation of cells that secrete reactionary dentin.[148] Fibroblast growth factor (FGF), transforming growth factor-beta (TGF-β), bone morphogenic protein (BMP) and vascular endothelial growth factor (VEGF) have all been shown to upregulate the differentiation of new odontoblast-like cells.[149-152] In order to harvest the potential of these molecules for regenerative endodontic procedures, EDTA is used to demineralize the dentin and release sequestered growth factors.[121]

RECOMMENDED CLINICAL CONSIDERATIONS FOR REPs

The AAE has published clinical considerations for a regenerative procedure which was revised on 4/1/2018.[32] The guidelines are as follows:

Case Selection

- Tooth with necrotic pulp and an immature apex
- Pulp space not needed for post & core, final restoration

- Compliant patient/parent
- Patient is not allergic to medicaments and antibiotics necessary to complete procedure (ASA 1 or 2)

  Informed Consent
- Two (or more) appointments
- Use of antimicrobial(s)
- Possible adverse effects: staining of crown/root, lack of response to treatment, pain/infection
- Alternatives: MTA apexification, no treatment, extraction (when deemed nonsalvageable)
- Permission to enter information into AAE database (optional)

  First Appointment
- Local anesthesia, dental dam isolation and access
- Copious, gentle irrigation with 20 mL NaOCl using an irrigation system that minimizes the possibility of extrusion of irrigants into the periapical space (e.g., needle with closed end and side-vents, or EndoVac™). Lower concentrations of NaOCl are advised [1.5% NaOCl (20 mL/canal, 5 min) and then irrigated with saline or EDTA (20 mL/canal, 5 min), with irrigating needle positioned about 1 mm from root end, to minimize cytotoxicity to stem cells in the apical tissues.
- Dry canals with paper points
    - Place calcium hydroxide or low concentration of triple antibiotic paste. If the triple antibiotic paste is used:
- Consider sealing pulp chamber with a dentin bonding agent (to minimize risk of

staining), and

- Mix 1:1:1 ciprofloxacin: metronidazole: minocycline to a final concentration of 1-5 mg/mL. Triple antibiotic paste has been associated with tooth discoloration. Double antibiotic paste without minocycline paste or substitution of minocycline for other antibiotic (e.g., clindamycin; amoxicillin; cefaclor) is another possible alternative as root canal disinfectant. Clinicians should be aware that studies have been done using higher concentrations of TAP/DAP, but a recommendation to a higher concentration can't be made at this time due to limited studies.
- Deliver into canal system via syringe
- If triple antibiotic is used, ensure that it remains below CEJ (minimize crown staining)
- Seal with 3-4mm of a temporary restorative material such as Cavit™, IRM™, glass-ionomer or another temporary material. Dismiss patient for 1-4 weeks.

Second Appointment (1-4 weeks after 1st visit)

- Assess response to initial treatment. If there are signs/symptoms of persistent infection, consider additional treatment time with antimicrobial, or alternative antimicrobial.
- Anesthesia with 3% mepivacaine without vasoconstrictor, dental dam isolation
- Copious, gentle irrigation with 20ml of 17% EDTA
- Dry with paper points
- Create bleeding into canal system by over-instrumenting (endo file, endo explorer) (induce by rotating a pre-curved K-file at 2 mm past the apical foramen with the goal of having the entire canal filled with blood to the level of the

cemento–enamel junction). An alternative to creating of a blood clot is the use of platelet-rich plasma (PRP), platelet rich fibrin (PRF) or autologous fibrin matrix (AFM).

- Stop bleeding at a level that allows for 3-4 mm of restorative material
    - Place a resorbable matrix such as CollaPlug™, Collacote™, CollaTape™ over the blood clot if necessary and white MTA as capping material
    - A 3–4 mm layer of glass ionomer (e.g. Fuji IX™, GC America, Alsip, IL) is flowed gently over the capping material and light-cured for 40 seconds. MTA has been associated with discoloration. Alternatives to MTA (such as bioceramics or tricalcium silicate cements [e.g., Biodentine®, Septodont, Lancasted, PA, USA, EndoSequence® BC RRM-Fast Set Putty, Brasseler, USA]) should be considered in teeth where there is an esthetic concern.

- Anterior and Premolar teeth - Consider use of Collatape/Collaplug and restoring with 3mm of a nonstaining restorative material followed by bonding a filled composite to the beveled enamel margin
- Molar teeth or teeth with PFM crown - Consider use of Collatape/Collaplug and restoring with 3mm of MTA, followed by RMGI, composite or alloy

Follow-up (6-, 12-, 24-months)

- Clinical and Radiographic exam
    - No pain, soft tissue swelling or sinus tract (often observed between first and second appointments)

    - Resolution of apical radiolucency (often observed 6-12 months after treatment)
    - Increased width of root walls (this is generally observed before apparent increase in root length and often occurs 12-24 months after treatment)
    - Increased root length
    - Positive pulp vitality test response
    - Recommended yearly follow-up after the first 2 years
    - CBCT is highly recommended for initial evaluation and follow-up visits

- The degree of success of Regenerative Endodontic Procedures is largely measured by the extent to which it is possible to attain primary, secondary, and tertiary goals:
    - Primary goal: The elimination of symptoms and the evidence of bony healing
    - Secondary goal: Increased root wall thickness and/or increased root length (desirable, but perhaps not essential)
    - Tertiary goal: Positive response to vitality testing (which if achieved, could indicate a more organized vital pulp tissue)

MATERIALS AND METHODS

TREATMENT GROUPS

Group 1 – Methylcellulose (MC)

Group 2 – MC + 1 mg/mL DAP

Group 3 – MC + 1 mg/mL DAP + 0.5% HA

Group 4 – MC + 1 mg/mL DAP + 1.0% HA

Group 5 – $Ca(OH)_2$

Group 6 – Media

Groups 1 and 6 served as negative controls. Group 5 served as a positive control as the effects of $Ca(OH)_2$ are known on DPSC growth.[153] UltraCal™ from Ultradent was used as the brand of $Ca(OH)_2$. Groups 2-4 serve as the experimental groups testing the effects of the medicaments on DPSC differentiation and mineral deposition. Based on these findings, we will use 0.5% and 1.0% HA as our two experimental concentrations for the test samples and controls. These groups are shown in Figure 2.

MATERIALS

Materials necessary for this experiment were purchased commercially, obtained from a previous experiment, or prepared using the methodology outlined below.

- 24-well cell culture plates (Alkali Scientific Inc. Cat: TP9024)
- DPSCs from immature third molars (Cook General Biotechnology, Indianapolis, IN, USA)
- Transwell chambers (Falcon Product 1.0 μm permeable support transmembrane with transparent PET membrane: #353104)
- Alpha modified minimal essential media (HyClone Laboratories Inc., South Logan, UT, USA)

- Fetal bovine serum (FBS) (Atlanta Biologicals Inc., Flowery Branch, GA, USA)
- Penicillin-streptomycin (Life Technologies Corporation, Grand Island, NY, USA)
- 0.25% trypsin/EDTA (Life Technologies Corporation)
- Double antibiotic capsules (Ciprofloxacin USP 400 mg; Metronidazole USP 400 mg; 1:1) (Champs Pharmacy, San Antonio, TX, USA)
- Methylcellulose (Methocel® 60 HG, 28-30% methoxyl basis)
- Hydroxyapatite (Nanopowder <200 nm size, Aldrich: 677418-10G)
- ALP assay kit (Sigma Aldrich)
- Alizarin-S red reagents
- Pierce BCA Assay Kit (Thermo Scientific: 23225)
- Cell culture grade water (Corning, Mediatech, Inc., Manassas, VA, USA)

METHODOLOGY

PREPARATION OF MC CONTAINING DAP ± HA

MC containing DAP was prepared using a previously published laboratory protocol.[27] A concentration of 1 mg/mL DAP was made by dissolving 25 mg of DAP in 25 mL cell culture grade water using sterilized glassware. Sterilized weighing dishes were used to measure DAP powder which was slowly added to the beaker in increments. Using a stir bar, the solution was mixed at speed 6 for 10 minutes until DAP was fully incorporated into a homogenous solution. For the groups with HA, HA nanoparticles were added to the solutions with sterilized weighing dishes at 0.5% or 1.0% (% = g/vol) and sonicated for 30 minutes. These groups were then stirred for 30 minutes. To achieve a paste-like consistency of the solutions, 2.0 g of MC powder was weighed using

sterilized weighing dishes and added slowly in increments to each solution. The gels were transferred to sterile 50 mL conical tubes and stored at 4°C until needed.

HUMAN DENTAL PULP STEM CELLS (DPSCs)

Previously obtained DPSCs were used for this study (Cook General Biotechnology, Indianapolis, IN, USA). The cells were stored in aliquots in liquid nitrogen tanks in the laboratory of Dr. Bruzzaniti. Cells were cultured with alpha MEM supplemented with 10% FBS and streptomycin/penicillin. Sub-confluent cells at approximately 80% confluence were detached from the culture plate using 0.05% trypsin-EDTA. Prior to each experiment, cells were thawed, passaged one time, and plated at 2 $x10^4$ cells per well in 24-well plates and incubated at 37ºC 5% CO_2 in culture media for 24 hours before adding medicaments.

Treatment medicaments and controls were added to DPSCs in 24-well plates on Day 0 as follows (Figure 1). First, media was aspirated and replenished with 500 μL fresh media, followed by placement of the transwells in the 24-well plate. Next, 750 μL media was added on top the transwells followed by 100 μL of each medicament group using a tuberculin syringe (Figure 3). The treatment plate was placed in the incubator for 3 days at 37°C, 5% CO_2. On Day 3, transwells containing medicaments and controls were removed and discarded, media was replaced with the addition of 10 mM β-glycerolphosphate (β-GP) and 50 mg/mL ascorbic acid (AA) to promote osteogenic differentiation. DPSCs were cultured for an additional 9 days with media being added approximately every 3 days. At Day 12, DPSCs were harvested for alkaline phosphatase activity and mineral deposition as described below.

QUANTIFICATION OF ALKALINE PHOSPHATASE ACTIVITY

Alkaline phosphatase (ALP) was quantified using the Sigma-Aldrich assay per previously published research from Dr. Bruzzaniti's laboratory.[30] After culture of DPSCs with medicaments or controls, the cells were washed with phosphate buffered saline (PBS) and stored at -20°C until assayed for ALP activity.

ALP lysis buffer was prepared as follows: 10 mL lysis buffer consisting of 7.92 mL dd-H_2O, 0.5 mL 1 M Tris-Cl (pH 7.5), 0.3 mL 5 M NaCl, and 1 mL 10% Igepal-CA 630 (NP-40). Protease and kinase inhibitors to prevent ALP degradation were prepared as follows: 0.25 mL of 10% sodium deoxycholate, 0.01 mL of 10 mg/mL leupeptin hydrochloride, 0.01 mL of 10 mg/mL aprotinin, and 0.01 mL of 10 mg/mL of pepstatin. Substrate solution was prepared as follows: 10 mL dd-H_2O mixed with 10 mL alkaline buffer and 40 mg 4-nitrophenyl phosphate sodium salt hexahydrate powder. Last, 40 µL NaOH (10 M) was mixed with 20 mL H_2O to produce 20 mM NaOH.

To prepare the cell lysate, DPSCs stored at -20 °C received 100 µL lysis buffer added to each well. DPSCs were scraped with a sterile pipette tip, and solutions were placed in microcentrifuge tubes. Cells were sonicated for 5 minutes and centrifuged on 13.2 x 10^3 RPM for 5 minutes. Supernatant was collected, labeled and stored at -34°C until ALP assay per the protocol below.

To assess ALP activity, the colorimetric conversion hydrolyzing p-nitrophenol phosphate to p-nitrophenol was performed according to protocols routinely used in Dr. Bruzzaniti's laboratory.[154] A standard curve was created using serial dilution. 200 µL of standards and 5 µl of cell lysate were added in triplicates to a 96-well plate. 100 µL of the

substrate solution (40 mg of p-nitrophenyl phosphate (p-NPP), 10 mL of alkaline buffer, and 10 mL of double distilled H_2O) was added to each well containing the treatment groups. The 96-well plate was covered in aluminum foil and incubated at 37°C for 1 hour. Colorimetric conversion of p-NPP to nitrophenol was used to determine when the treatment groups are within the color range of the standards. After 1 hour, the reaction was stopped by adding 95 µl of 20 mM NaOH to the treatment groups. The plate was allowed to cool to room temperature and a plate reader was used to measure optical absorbance at 405 nm (Figure 4).

Total protein in the cell lysates was used to normalize ALP activity. The Pierce BCA Assay kit (Thermo Scientific) was followed according to the manufacturer's protocol. A working solution of BSA 250 µg/mL in dH_2O was made to generate the standard curve. 10 µL of the protein lysates were added to the BCA reaction mixture in duplicates. Samples were vortexed and incubated at 60°C for 1 hour. The plate was allowed to cool to room temperature and a plate reader was used to measure optical absorbance at 562 nm. Total protein in each lysate was determined based on the BSA standard curve. This assay was performed once with 4 samples per group, and the samples were run in triplicate.

MINERAL DEPOSITION ASSAY USING ALIZARIN RED S

To assess mineral deposition by DPSCs, the cells were cultured, treated, and differentiated with β-glycerolphosphate and ascorbic acid prior to assay as outlined above. The Alizarin Red S assay was performed essentially as reported in published research from Dr. Bruzzaniti's laboratory.[30] Treatment groups were added on Day 0 and

media were replenished approximately every 3 days as previously outlined. On Day 12, DPSCs were washed with phosphate buffered saline (PBS), fixed with 500 μL 3.7% formaldehyde in PBS for 15 minutes, and washed again with PBS prior to storing at 4°C prior to assay.

Alizarin Red S solution (40 mM in H_2O, pH 4.1) and 1% cetylpyridinium chloride (CPC) were gathered. Alizarin Red S solution was mixed with a stir bar and a standard curve was prepared in 1.7 mL microcentrifuge tubes based on the known ability of calcium binding to Alizarin Red S (2 mol of Ca2+/mol Alizarin Red S).[155] PBS was removed from the fixed mineral deposition 24-well plate and the cells were rinsed twice with dH_2O. 500 μL 40 mM Alizarin Red S was added to each well and the plate was placed on the shaker at speed 20 for 15 minutes. Alizarin Red S was aspirated and the fixed cells were rinsed four times with H_2O. H_2O was aspirated and cells were washed with 1 mL of PBS followed by placement on the shaker at speed 20 for 15 minutes. PBS was aspirated and 500 μL CPC was added to each well to extract Alizarin Red S. The plate was placed on the shaker at speed 20 for 15 minutes. For analysis, 150 μL of standards and samples were transferred to a 96-well plate in triplicates and optical absorbance was measured at 562 nm. This assay was performed once with 4 samples per group, and the samples were run in triplicate (Figure 5).

SAMPLE SIZE AND STATISTICAL METHODS

One-way analysis of variance (ANOVA) was used to compare the groups for differences in ALP activity and mineral deposition, with different variances allowed for each group and a random effect included in the model to account for correlation within

each of the trials. One trial with 4 samples per group were conducted. Pair-wise comparisons were made using Tukey's multiple comparisons procedure to control the overall significance level at 5%. All analyses were performed using SAS version 9.4 (SAS Institute, Inc., Cary, NC, USA). Bar graphs show the mean of 4 wells/treatment group with standard error of the mean.

RESULTS

RESULTS

THE EFFECT OF HA MEDICAMENTS ON ALP ACTIVITY IN DPSCs

To assess osteogenic differentiation, we assessed the activity of ALP, which is activated early during matrix maturation.[156] As shown in Figure 6, the two treatment groups containing DAP and HA (DAP + 0.5% HA + MC, DAP + 1.0% HA + MC) showed a trend towards increased ALP activity over all other treatment groups. This difference was not statistically significant, however. All other groups showed similar ALP activity. These results are outlined in Tables 1 and 2.

THE EFFECT OF HA MEDICAMENTS ON MINERAL DEPOSITION BY DPSCs

The two treatment groups containing DAP and HA (DAP + 0.5% HA + MC, DAP + 1.0% HA + MC) showed a trend towards greater amount of mineral deposition compared to DAP + MC as well as the MC and media controls (Figure 7). These differences were not statistically significant. Further, although no statistical difference was found between $Ca(OH)_2$ and the other treatment groups, the $Ca(OH)_2$ appeared to show highest level of mineral deposition. It also appears that the addition of HA may improve mineral deposition compared to the DAP + MC group, albeit this was not statistically significant. These results are outlined in Tables 1 and 3.

FIGURES AND TABLES

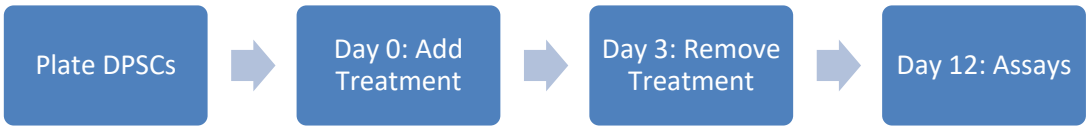

FIGURE 1. Flowchart containing experimental design.

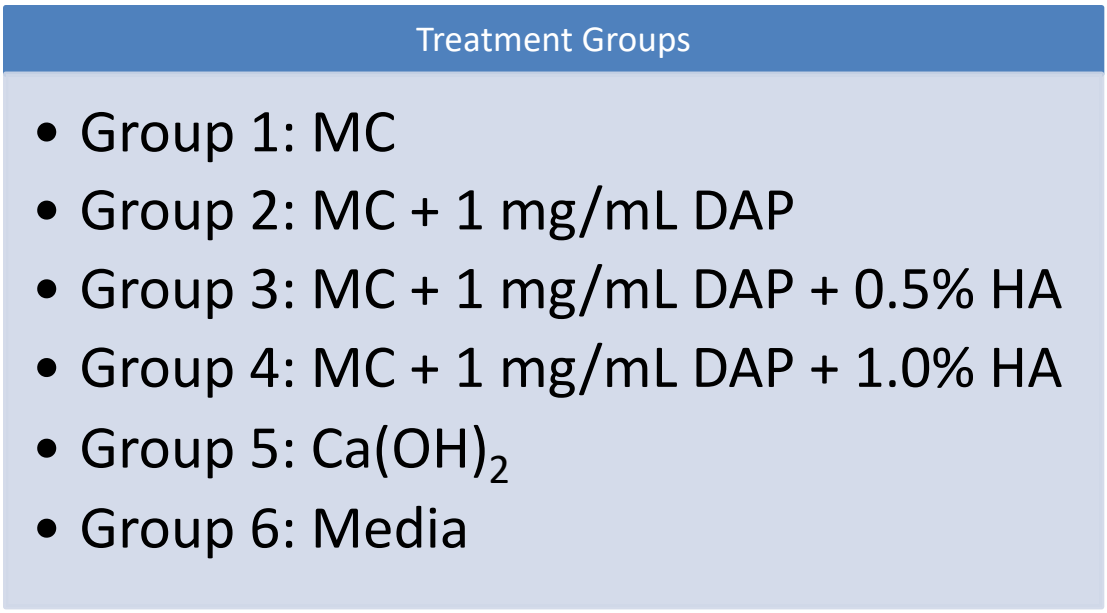

FIGURE 2. Treatment groups.

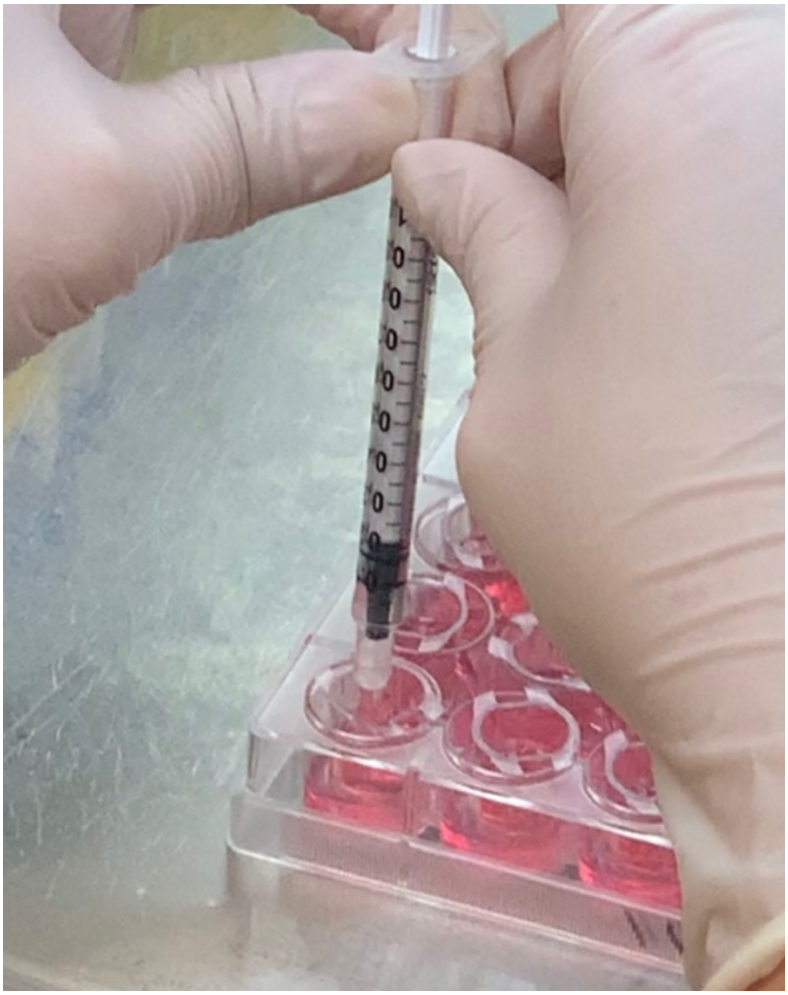

FIGURE 3. Medicaments added with tuberculin syringe.

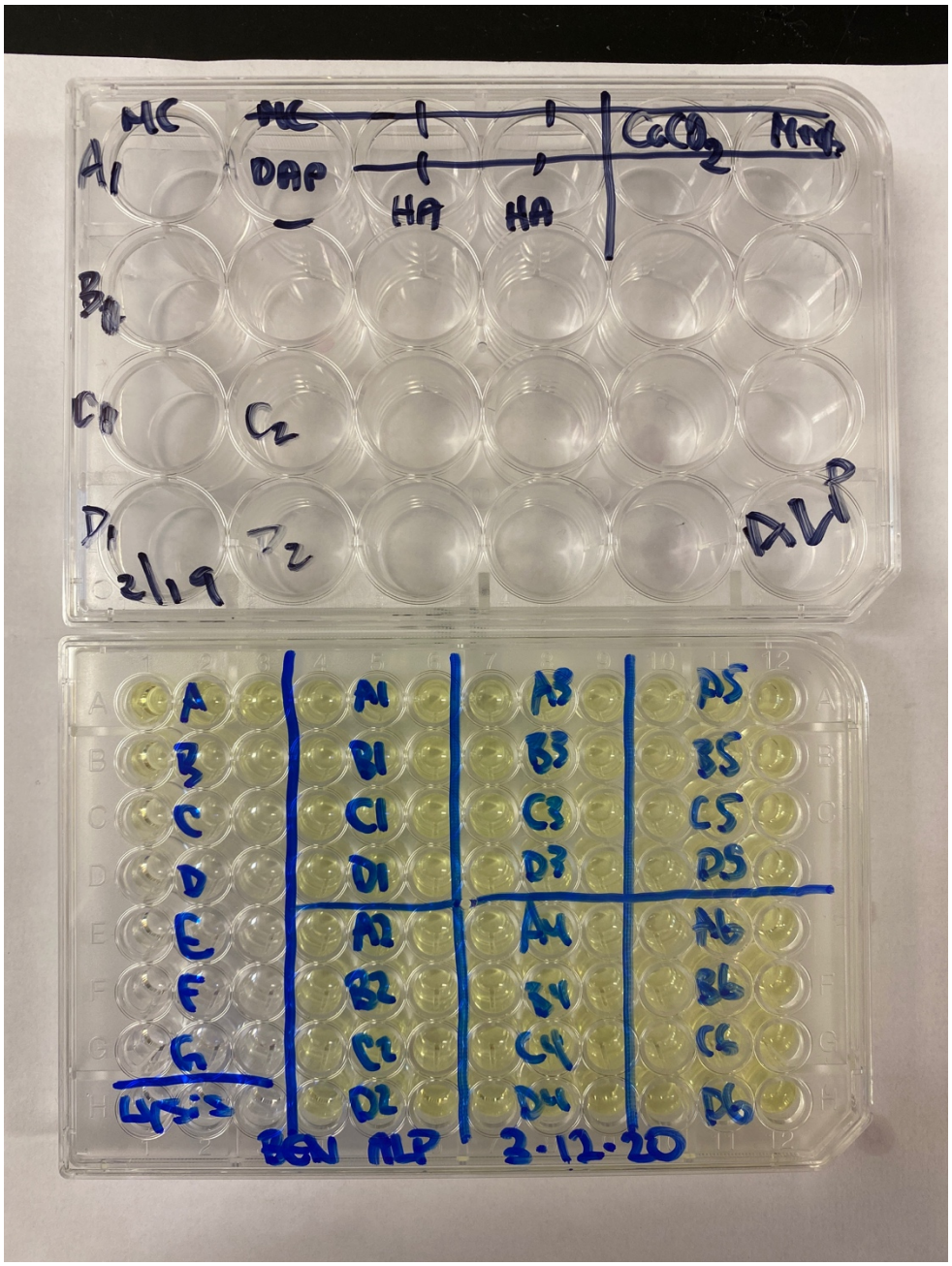

FIGURE 4 ALP assay.

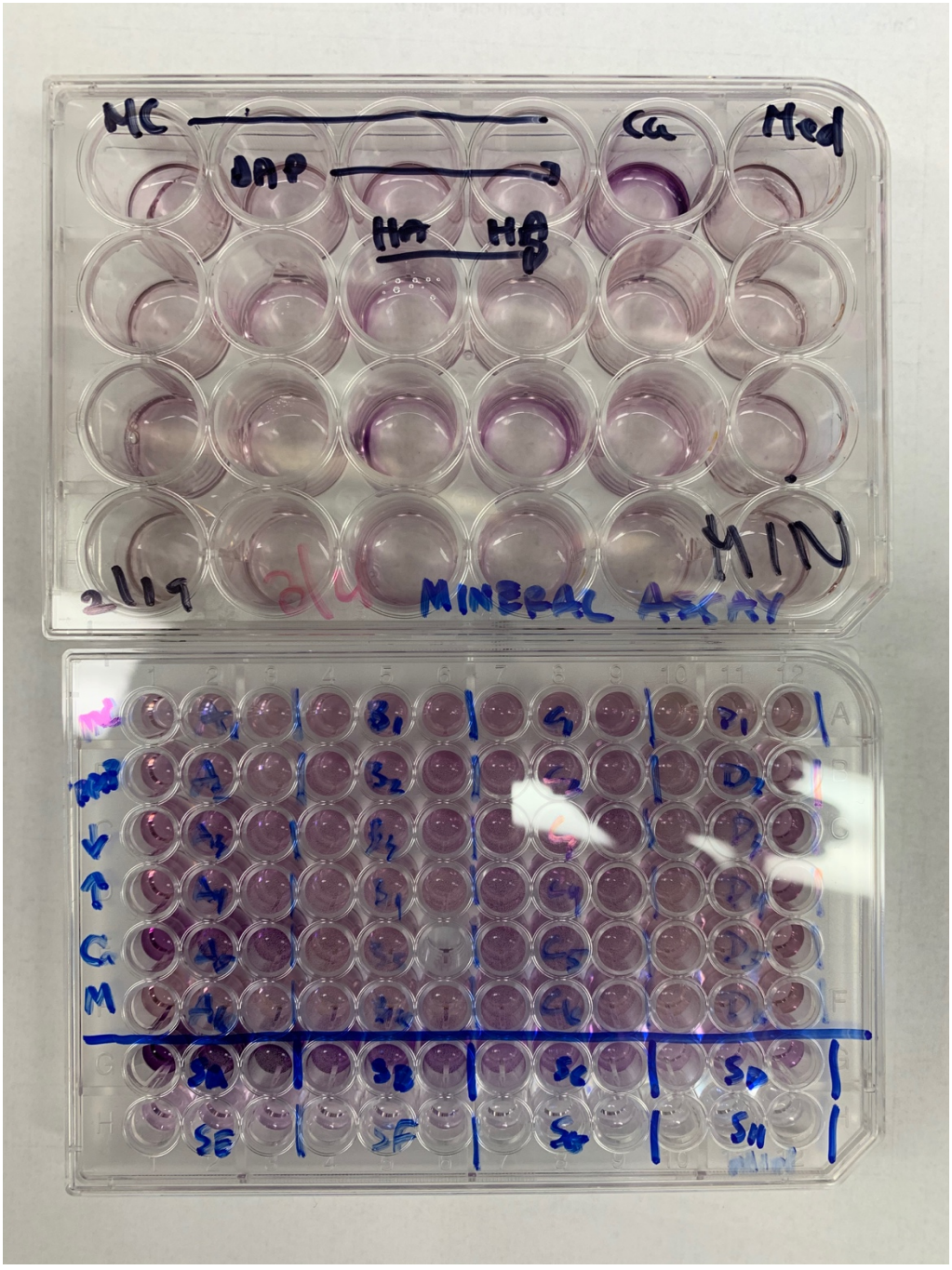

FIGURE 5. Mineral deposition assay.

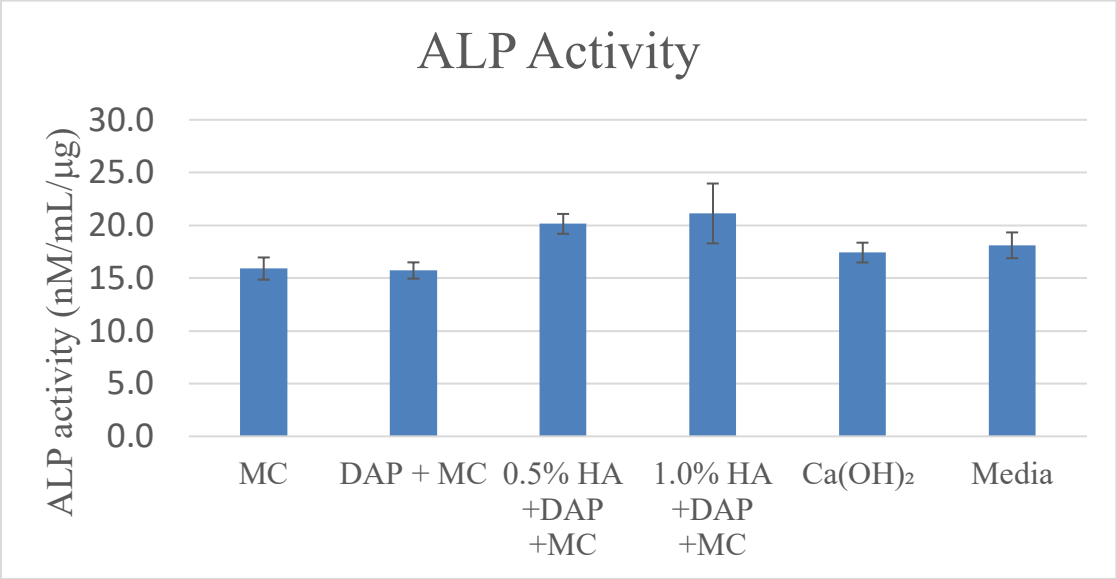

FIGURE 6. ALP results showing the mean of 4 wells/treatment group with SEM.

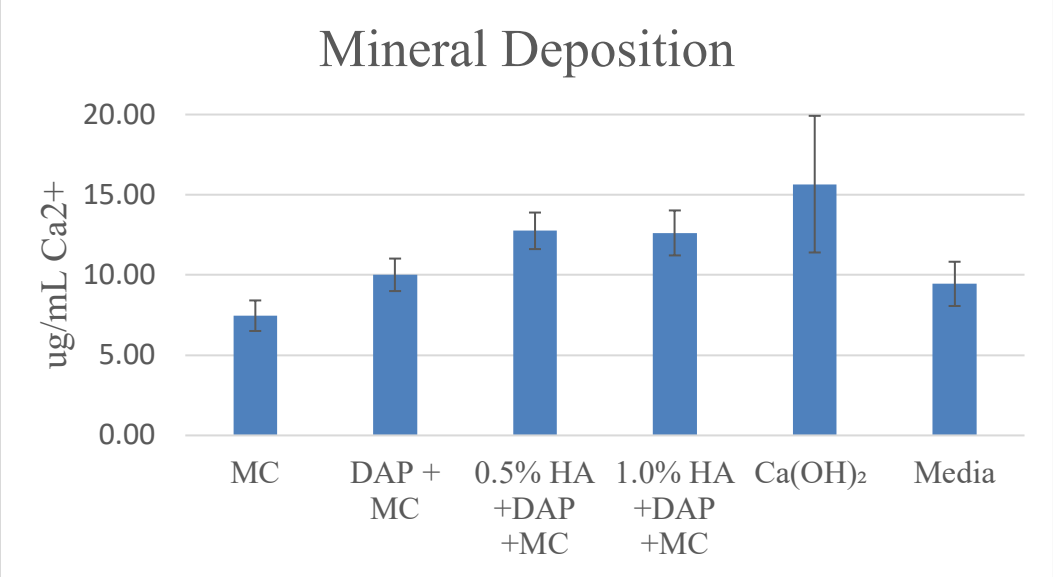

FIGURE 7. Mineral deposition results showing the mean of 4 wells/treatment group with SEM.

TABLE I

Assay results

Outcome	Group	N	Mean	SD	SE	Min	Max
ALP activity (nM/mL/μg)							
	$Ca(OH)_2$	4	17.41	1.88	0.94	15.76	20.12
	DAP + MC	4	15.72	1.54	0.77	13.69	17.40
	1.0% HA + DAP + MC	4	21.12	5.67	2.84	15.39	28.85
	0.5% HA + DAP + MC	4	20.14	1.88	0.94	17.58	22.08
	MC	4	15.90	2.10	1.05	12.75	17.03
	Media	4	18.11	2.44	1.22	14.58	20.09
Mineral deposition (ug/mL Ca2+)	$Ca(OH)_2$	4	15.66	8.52	4.26	8.63	27.37
	DAP + MC	4	10.01	2.03	1.01	7.97	12.12
	1.0% HA + DAP + MC	4	12.62	2.80	1.40	9.65	16.41
	0.5% HA + DAP + MC	4	12.75	2.28	1.14	10.23	15.56
	MC	4	7.46	1.90	0.95	5.05	9.57
	Media	4	9.45	2.76	1.38	7.73	13.53

TABLE II

Pair-wise comparisons – ALP activity (nM/mL/μg)

Result	**p-value**
$Ca(OH)_2$ & DAP + MC n.s.	0.961
$Ca(OH)_2$ & 1.0% HA + DAP + MC n.s.	0.500
$Ca(OH)_2$ & 0.5% HA + DAP + MC n.s.	0.777
$Ca(OH)_2$ & MC n.s.	0.976
$Ca(OH)_2$ & Media n.s.	0.999
DAP + MC & 1.0% HA + DAP + MC n.s.	0.149
DAP + MC & 0.5% HA + DAP + MC n.s.	0.319
DAP + MC & MC n.s.	1.000
DAP + MC & Media n.s.	0.855
1.0% HA + DAP + MC & 0.5% HA + DAP + MC n.s.	0.997
1.0% HA + DAP + MC & MC n.s.	0.173
1.0% HA + DAP + MC & Media n.s.	0.698
0.5% HA + DAP + MC & MC n.s.	0.362
0.5% HA + DAP + MC & Media n.s.	0.919
MC & Media n.s.	0.891

TABLE III

Pair-wise comparisons – Mineral deposition (ug/mL Ca2+)

	p-value
$Ca(OH)_2$ & DAP + MC n.s.	0.407
$Ca(OH)_2$ & 1.0% HA + DAP + MC n.s.	0.895
$Ca(OH)_2$ & 0.5% HA + DAP + MC n.s.	0.911
$Ca(OH)_2$ & MC n.s.	0.098
$Ca(OH)_2$ & Media n.s.	0.310
DAP + MC & 1.0% HA + DAP + MC n.s.	0.941
DAP + MC & 0.5% HA + DAP + MC n.s.	0.929
DAP + MC & MC n.s.	0.947
DAP + MC & Media n.s.	1.000
1.0% HA + DAP + MC & 0.5% HA + DAP + MC n.s.	1.000
1.0% HA + DAP + MC & MC n.s.	0.502
1.0% HA + DAP + MC & Media n.s.	0.877
0.5% HA + DAP + MC & MC n.s.	0.476
0.5% HA + DAP + MC & Media n.s.	0.858
MC & Media n.s.	0.981

DISCUSSION

The primary goal of regenerative endodontic procedures is disinfection of the infected root canal system, either through the use of $Ca(OH)_2$ or antibiotic paste. While both medicaments have been used with much success, Sabrah et al. found that TAP and DAP significantly decreased biofilm formation compared to $Ca(OH)_2$.[23] Sabrah et al. helped shape the AAE guidelines, specifically in relation to antibiotic concentration used during the initial visit. Currently, a recommended concentration of 1-5 mg/mL of TAP or DAP is advised. This is partly based previously published studies at this university finding that concentrations from 0.125 to 10 mg/mL of TAP and DAP showed a significant direct antibacterial effect against *E. faecalis* biofilm.[20] Additional studies found TAP and DAP to have residual antibacterial effects when radicular dentin was treated with antibiotic paste.[157] Interestingly, the authors found DAP had a more pronounced residual antibacterial effect than TAP at all tested concentrations.[157] Other studies found that dentin pretreated with DAP at concentrations of 5 mg/mL or higher showed significant residual antibacterial effects regardless of treatment time,[158, 159] with direct antibacterial effects as low as 1 mg/mL.[159] Another study showed that 10 mg/mL of TAP produced significant direct and residual antibacterial action against *E. faecalis* biofilm.[29] While these concentrations appear to exhibit successful antibacterial properties, in regenerative endodontic procedures the aim is to disinfect the root canal system while minimizing cytotoxicity to stem cells. Thus, it is important to balance the role of disinfecting the root canal system with lower concentrations of antibiotic paste that are not cytotoxic to DPSCs.[20]

Additional interest has focused on the role of MC as a delivery vehicle and scaffolding agent for lower concentrations of antibiotic paste. The benefit of using MC is

that it can be used as a delivery vehicle for low concentration of antibiotic while ensuring satisfactory handling characteristics.[29] The addition of MC to TAP has been shown to permit a lower concentration of TAP, thereby minimizing the reduction in microhardness of roots compared to higher concentrations of TAP.[28] While the addition of MC may improve clinical outcomes, its placement cannot be confirmed radiographically as is possible for $Ca(OH)_2$. To overcome this issue, radiopaques have been added to antibiotic pastes to permit visualization on dental radiographs. One study found that $BaSO_4$ DAP at 1 mg/mL provided significantly superior residual antibacterial effects and comparable radiopacity to $Ca(OH)_2$.[31] Another study that was recently published sought to quantify the effect of adding radiopaques to DAP with MC on DPSCs. The authors found that the addition of $BaSO_4$ or ZrO_2 did not adversely affect dental pulp stem cell proliferation.[160] In fact, they found that low DAP concentrations and short exposure times favored increased ALP concentration and mineral deposition of DPSCs.[160]

These same researchers also studied the antimicrobial properties and cytotoxic effects of a DAP loaded MC carrier on DPSCs.[30] They found that DAP at 1, 5 and 10 mg/mL produced significant direct antibacterial effects against single and dual species biofilms. Similar to other studies from this institution, the authors also found that DAP hydrogel at a concentration of 5 mg/mL or higher is required for a residual antibacterial effect.[30] At 1 mg/mL, however, no residual antibacterial effect was noted. Interestingly though, 1 mg/mL was the only concentration that did not have a significant negative effect on DPSC proliferation, ALP activity and mineral deposition.[30]

Expanding from these findings, an unpublished study from the laboratory of Dr. Angela Bruzzaniti sought to investigate the addition of HA to a DAP loaded MC carrier

on the cytotoxicity and differentiation potential of DPSCs. Experimental concentrations of HA were 0.25%, 0.5% and 1.0%. The addition of HA aimed to provide additional scaffold and stimulate hard tissue growth through osteoinductive action on osteoblasts.[39] The investigators found all groups containing DAP, as well as the $Ca(OH)_2$ control, showed robust metabolic activity using an MTS assay, suggesting that proliferation was increased. Therefore, they concluded that the addition of HA did not negatively impact dental pulp stem cell proliferation. When assessing ALP activity, MC+DAP+0.5% HA and MC+DAP+1.0% HA produced the greatest response. These two groups showed a statistically significant increase in ALP activity over all other groups, with the exception of the experimental group with the lowest concentration of HA (MC+DAP+0.25% HA).[45] Thus, the author remarked that the addition of HA significantly increased the cells' ability to differentiate into an osteogenic lineage. The author also sought to compare the experimental groups on their ability to induce mineral deposition of DPSCs. Much like to the proliferation assay, the groups all produced similar results with the exception of MC+DAP+0.5% HA, which showed a statistically significant greater response than MC+DAP.

These unpublished findings served as the basis for the current study. Our study sought to assess the differentiation and mineral deposition of DPSCs using most of the same experimental groups as well as a media control, but with extended culture times to potentially increase osteogenic differentiation in our DPSC cultures. Therefore, we treated DPSC with medicaments for 3 days, followed by post-treatment culture conditions without medicaments for 9 days, prior to assay of ALP activity and mineral deposition. Our study demonstrated that ALP activity was most pronounced in the

MC+DAP+0.5% HA and MC+DAP+1.0% HA groups after 9 days post-treatment. The results were not significant, due to insufficient sample size and an inability to complete further tests. Nevertheless, these data indicate that the experimental groups may increase ALP activity, similar to the findings of Drs. Everhart and Bruzzaniti. Interestingly, although our findings were not statistically significant, we were able to obtain the same trend towards increased ALP activity after a shortened medicament exposure time of 3 days in our study, compared with 7 days in the Everhart study.

When evaluating the mineral deposition assay results, $Ca(OH)_2$ appeared to produce the greatest mineralized nodule formation. Although this was not statistically significant, this trend is similar to what has been found previously by McIntyre et al.[30] Calcium ions release from $Ca(OH)_2$ have been previously shown to play an important role in mineralization by promoting cellular migration and differentiation of DPSCs[161]. The MC+DAP+0.5% HA and MC+DAP+1.0% HA groups in our study produced greater mineral deposition than the MC+DAP, MC, and media groups. These trends imply HA can may be useful as an additional osteogenic agent when added to MC+DAP.

This study provides additional support that low concentration DAP+MC may have application to regenerative endodontic procedures. The addition of HA may provide additional osteogenic potential. Although the sample size was small, the trends found in the current study warrant consideration and further study. Taken together, our results imply that with a shorter exposure time, differentiation and mineral deposition of DPSCs may be attained. Although a 3-day treatment time is less than currently recommended by the AAE, perhaps future research into the pharmacokinetics of drug delivery could design a system with a gradual release of antibiotic and scaffold over time. This example would

degrade slowly over time to provide sustained antibacterial action while permitting a lower concentration of antibiotic that is permissible to stem cell viability and osteogenic differentiation.

SUMMARY AND CONCLUSIONS

In conclusion, given the insufficient sample size of the current study, it is not possible to conclusively support or reject the null hypothesis, which stated that the addition of HA nanoparticles to a DAP loaded MC carrier will have no effect on the differentiation and mineral deposition of DPSCs over time. Additional future studies are warranted to achieve a sample size of nine, which is based on our sample size calculation. Nevertheless, the addition of HA to MC+DAP showed a trend towards improved differentiation and mineral deposition of DPSCs after 3-days of exposure to the medicaments. These results showed that even with a shortened treatment time, increased differentiation and mineral deposition of DPSCs may be possible.

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ABSTRACT

THE IMPACT OF HYDROXYAPATITE ON ALKALINE PHOSPHATASE ACTIVITY AND MINERAL DEPOSITION OF DENTAL PULP STEM CELLS USING A DOUBLE ANTIBIOTIC PASTE LOADED METHYLCELLULOSE CARRIER

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Introduction: Regenerative endodontic procedures (REPs) are a type of endodontic treatment aimed at replacing damaged tooth structures, including dentin and root structures, as well as cells of the pulp-dentin complex. Double antibiotic paste (DAP) has been shown to be efficacious in achieving disinfection of the root canal system while minimizing cytotoxicity to dental pulp stem cells (DPSCs). Hydroxyapatite (HA) is an extracellular, mineralized component of bone that has shown much promise as a scaffold in the field of regenerative medicine.

Objective: The objective of this study was to evaluate the effects of HA in a DAP loaded methylcellulose (MC) carrier on the differentiation and mineral deposition of

DPSC over time.

Materials and Methods: DPSCs were plated in 24-well plates with culture media. The following day, semi-permeable 0.1 μm chambers were inserted into the wells to separate the reservoirs and permit delivery of medicaments. 100 μL treatment paste composed of MC with 1% DAP and either 0.5% or 1.0% nano-HA was added, followed by additional culture media. After 3 days of treatment, medicaments were removed and DPSCs were cultured for an additional 9 days with replacement of media every 3-4 days. At Day 12, DPSCs were evaluated for alkaline phosphatase (ALP) activity using a biochemical assay and mineral deposition using an Alizarin Red S Ca2+ staining assay (4 wells/group). Comparisons between groups were performed using one-way analysis of variance (ANOVA) with a 5% significance level used for all tests.

Results: A trend towards increased ALP and mineral deposition activity was noted among the groups with HA added to DAP with MC. Although these trends were not statistically significant, a trend towards increased ALP and mineral deposition was observed after 3-day medicament exposure. The results were similar to previous findings using 7-day medicament treatments.

Conclusion: The addition of HA showed a trend towards improved differentiation and mineral deposition of DPSCs compared to DAP with MC. Although additional studies are required, these results showed suggest that even with a shortened treatment time, increased differentiation and mineral deposition of DPSCs may be possible. This study provides additional support that low concentration DAP in a MC carrier has potential application in regenerative endodontic procedures. The novel addition of HA may provide additional osteogenic potential.

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